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Sexual dimorphism of arrestment and gregariousness in the bed bug (*Cimex lectularius*) in response to cuticular extracts from nymphal exuviae

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Abstract. Releaser pheromones have direct behavioural effects to arrest, attract or disperse insects, whereas interactions within groups of social insects are often influenced by primer pheromones. The behaviour of insects displaying intermediate levels of sociality is largely unexplored in this context. In the present study, both the gregariousness and arrestment (settling near the odour source) of bed bugs *Cimex lectularius* L. (Hemiptera: Cimicidae) in response to conspecific exuvial extracts are described. Adult males are arrested on filter papers with extracts derived from exuviae of fifth-instar nymphs. Adult females and nymphs display no significant evidence for such behaviour. Adults of both sexes show no preference for extracts of male versus female fifth-instar exuviae. Arrestment of adult males does not occur on papers treated with fourth-instar exuvial extracts. Because the insects are assayed behaviourally in groups, an index is calculated describing how far bugs are away from being located independently of one another, as a measure of gregariousness. Adult males have lower values for this index (i.e. locations are closer to independence). Adult females, nymph cohorts and mixed age groups all have higher values for this index, which tend to increase over time. Females exhibit a clear increasing dose-dependent relationship for this index. It is concluded that the extracts of fifth-instar nymphal exuvia arrest males on refuges that possess the odour source. However, gregariousness is induced in females, without evidence of a tendency to assemble near the odour source.

Key words. Arrestment, cuticular hydrocarbons, gregariousness, primer pheromone, releaser pheromone.

Introduction

Over a broad range of insect taxa, many pheromones are linked to distinct behaviours, including but not limited to mate location (Dawson *et al*., 1990; Roelofs, 1995; Leal, 1997; Ando *et al*., 2004) and aggregation (Wertheim *et al*., 2005). Such signals are often described as 'releaser' pheromones if they are mediated entirely by the nervous system and evoke immediate behaviours (Wilson, 1965; Shorey, 1973). Pheromones are considered to have a 'primer' effect if a physiological alteration occurs that changes the behavioural repertoire (Wilson, 1965). Both the physiological and behavioural criteria provided by these definitions may not apply necessarily to all scenarios. Many pheromones classified clearly as primers exist in social insects, involving behaviours that promote group cohesion in social insects, such as the suppression of the sexual development or reproductive output of workers (Le Conte & Hefetz, 2008). Only rarely is it demonstrated clearly that more 'primitively social' insects utilize primer pheromones to moderate social interactions. One example is the desert locust *Schistocerca gregaria,* which has a pheromone that is an oviposition releaser, yet primes a shift to a gregarious physiological state (Malual *et al*., 2001).

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The common bed bug *Cimex lectularius* L. (Hemiptera: Cimicidae) is a species that exhibits primitive social behaviour. The adults and all nymphal stages are normally nocturnal feeders on humans (Usinger, 1966), although they can sometimes feed on other species that may be associated with humans, such as bats (Usinger & Beaucournu, 1967). When not feeding, bed bugs cluster together in large numbers in harbourages in secluded areas. These harbourages contain bugs of all stages that are in constant physical contact with each other, and with the remains of shed exoskeletons (exuviae) from developing bugs. Aside from the obvious benefit of remaining hidden, there is some evidence that aggregation can be beneficial for tolerating bouts of dehydration during periods of quiescence (Benoit *et al*., 2007). Aggregation behaviour is also linked to feeding status, with starved individuals being less likely to aggregate (Olson *et al*., 2009).

Two lines of inquiry are established with respect to bed bug chemical ecology: one involving volatile glandular-produced signals and the other involving the deposition of substrate contact pheromones. Both approaches lead to the description of behaviours typical of releaser pheromones. The adults of both sexes have a metathoracic scent apparatus (Kemper, 1929) known to contain (*E*)-2-hexenal and (*E*)-2-octenal (Schildknecht, 1964; Collins, 1968), which comprise components of an alarm pheromone (Levinson & Bar Ilan, 1971; Levinson *et al*., 1974b). Although nymphs have no metathoracic scent glands, they do have dorsal abdominal glands clearly visible on the cuticle of the third through fifth abdominal segments (Usinger, 1966). The glands of nymphs also contain volatile compounds, including the known alarm pheromones (Collins, 1968; Levinson *et al*., 1974a; Feldlaufer *et al*., 2010). Early research suggests that (*E*)-2-hexenal and (*E*)-2-octenal might act only as alarm pheromones (Levinson & Bar Ilan, 1971). However, a complex blend of these compounds and others found in the airspace surrounding harbourages attracts *C. lectularius* (Siljander *et al*., 2008).

Another line of inquiry involves 'trail' or 'contact' pheromones that are deposited on filter paper after exposure to bed bugs (Levinson & Bar Ilan, 1971). Filter papers exposed in this way possess large amounts of excretory material, and perhaps some material shed from the cuticular surfaces. The active components of such deposits are extractable in methanol but not in less polar solvents such a dichloromethane (Parashar *et al*., 2003), suggesting that cuticular hydrocarbons from the surface do not comprise the active component of such depositions. It is also demonstrated that methanol extracts such as these exhibit stage and sex specificity with respect to their arrestment effects (Siljander *et al*., 2007).

The haematophagous bug species *Triatoma infestans* assembles in response to its own faecal material (Lorenzo & Lazzari, 1996). However, aggregation in *T. infestans* is also promoted by cuticular compounds extractable in the nonpolar solvent hexane (Lorenzo Figueiras & Lazzari, 1998). Because *C. lectularius* are in constant contact with each other, and with the shed exuviae of nymphs at sites of aggregation, it is also plausible that they use cuticular compounds as semiochemicals. In the present study, the possible behavioural effects of dichloromethane extracts of nymphal cuticle obtained from freshly shed exuviae are examined. The behavioural responses to extractions are investigated for groups of bed bugs arranged in either uniformly-aged cohorts of nymphs, single sex groups of adults, or mixed groups consisting of male adults, female adults and nymph instars. By presenting the extracts to bugs in groups, a separation of the effects on arrestment (settling near the odour source) and gregariousness (settling near each other) is attempted in a biologically realistic context. A spatially-oriented arrestment response to the extracts would be typical of releaser pheromones, whereas gregariousness, as measured by nonrandom movement among the insects, would suggest a class of behaviour more similar to that induced by primer pheromones.

Materials and methods

Insects

The common bed bug *C. lectularius* was sourced from a colony in Beltsville, Maryland, which was established from bugs obtained from Harold Harlan (Crownsville, Maryland). The colony was kept at either ambient room temperature or in an incubator at 25–29 °C and 40–50% relative humidity. Bed bugs were fed weekly through a Parafilm® (VWR, Arlington Heights, Illinois) membrane on either packed red blood cells or packed red blood cells fortified with plasma (v/v, 1.25 : 1; Takano-Lee *et al*., 2003) obtained from the Walter Reed Army Medical Center (Washington, District of Columbia). During feeding, the red cells or red cells/plasma were maintained at 38 °C in a jacketed beaker (Kontes Glass Co., Vineland, New Jersey) attached to a recirculating water bath.

Chemical extracts

Exuviae from fourth- or fifth-instar nymphs were used in the study. Prior to eclosion, nymphs were removed from the main colony and placed in individual vials. This allowed the collection of exuviae from newly-moulted bugs that were minimally contaminated by excretions and/or other debris usually associated with exuviae collected from the main colony. It was also possible to establish the sex of the fifth-instar from which the exuvia derived by observing the emerging adult within each vial. The three dorsal abdominal scent glands were excised from the exuvia using micro-dissecting scissors. Signs of faecal contamination in the form of small dried blood droplets were also excised with scissors. Similarly, the hindguts were removed with any excretory material retained within them. The exuviae were then extracted in dichloromethane (Reagents, Inc.; Charlotte, North Carolina). A period of 24 h after the glands were removed was allowed to elapse before extraction to ensure full dissipation of any volatile compounds that might have been released when handling the bugs.

A 1-mL aliquot of the extract was analyzed by chromatography-mass spectrometry [7890A gas chromatograph/5975C mass spectrometer; Agilent Technologies, Santa Clara, California; equipped with an RTx 5MS column (30 m ×

0.25 mm inside diameter × 0.25 μm coating); Restek, Bellefonte, Pennsylvania] to verify the absence of alarm pheromones and the presence of compounds having mass spectra characteristic of cuticular hydrocarbons. The temperature was programmed for 50 °C for 2 min, increased to 280 °C at 10 °C min^{-1}, and then held at 280 °C for an additional 15 min. All spectra at each retention time were compared with those from authentic standards of pheromone gland constituents.

Choice arenas

Assay arenas consisted of glass crystallizing dishes (diameter 12.5, height 6.5 cm) with a piece of filter paper (diameter 9 cm) (Whatman No. 1; Whatman International Ltd, U.K.) placed in the centre of the dish (Fig. 1). Approximately 1 h before the experiment, smaller filter paper discs (diameter 4.25 cm) were fan-folded and treated with either the appropriate exuvial extract or a dichloromethane control. The treated fan-folded papers were placed on the 9-cm diameter filter paper directly across from each other, creating two refuges in each arena. All experiments were performed with ambient light from two shaded windows. The opposing refuges were always oriented parallel to this ambient light to minimize any potential effect of the light. The position of each arena was randomized at the initiation of an experiment, as were the positions of the exuviae-treated and solvent-treated filter papers.

Data collection and analysis

Each assay was performed with six insects. Between daily experiments, the experimental insects were kept together in these groups in Petri dishes lined with filter paper in an incubator at 27 ± 2 °C and 45 ± 5% relative humidity. Each day, the location of insects within each arena was measured four times (1, 2, 20 and 21 h after the experiment began). The time intervals were selected to establish how quickly the responses would occur, as well as to determine whether disruption during the course of measurement would affect the results. Furthermore, the long overnight acclimation period (2–20 h) is similar to that used in other bed bug studies (Siljander *et al*., 2007, 2008). All observations were made in the daytime: the first two being in the afternoon and the others the next morning. When making each observation, treated filter paper discs were quickly lifted with forceps to identify the *C. lectularius*, and then immediately restored to their previous positions. The numbers of *C. lectularius* in each of the refuges or elsewhere in the arena were recorded.

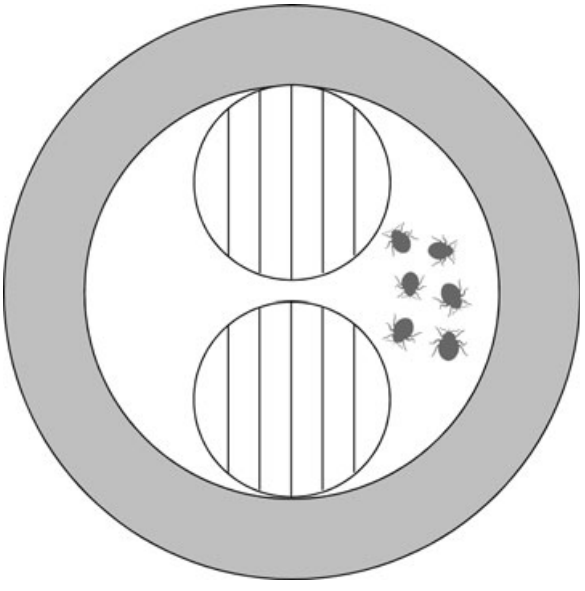

Fig. 1. Top view of a bioassay chamber. Outer ring shows the glass surface of crystallizing dish. A larger filter paper was centred in the arena, under a pair of fan-folded smaller filter papers, which were treated with extracts of exuviae or solvent. Insects (*Cimex lectularius*) were placed as shown at the start of each experiment.

Experiment 1: Instar and adult sex-specific behavioural responses to fifth-instar exuvial extracts

Each insect group was presented with the choice of fan-folded filter papers treated with either 20 μL of dichloromethane or 20 μL of a 10 exuviae mL^{-1} extract. This dose yields a 0.2 nymph-equivalent (NE). On two separate occasions, the extracts needed for this experiment were prepared from unsexed exuviae collected as described above. The extracts consisted of 20 exuviae in 2 mL of dichloromethane. Each extract from the exuviae was prepared immediately prior to application to the filter paper. Multiple extracts were performed for this and subsequent experiments, aiming to ensure that there were no large differences in the behavioural outcomes unique to a particular extraction.

Insects were fed 2 days before the assays of each cohort. Each day, the insect cohorts were assigned randomly to six arenas: first- and second-instar nymphs; third- and fourth-instar nymphs; fifth-instar nymphs; adult males; adult virgin females; and a combination of an adult male, an adult female and four nymphs (one from each of the second to fifth stadia). After six to eight single-day choice tests, new cohorts of *C. lectularius* were selected from the colony for each treatment and fed before being used in another series of experiments. Because other studies have demonstrated a decline in aggregation after 2 weeks of starvation (Olson *et al*., 2009), the protocol specified in the present study should have prevented a similar decline in such behavioural tendencies. The first two cohorts assayed were presented with filter paper treatments derived from the first exuvial extract. The last two cohorts were exposed to a second exuvial extract. In total, there were 28 replications of the choice assay for each of the insect treatments.

Experiment 2: Male and female adult responses to fifth-instar exuvial extracts of varying doses

Groups of *C. lectularius* of each sex were presented with filter paper refuges having a solvent control versus one of three doses of fifth-instar exuvial extract. The extracts were

prepared at respective proportions of 5, 25 or 50 exuviae mL^{-1} dichloromethane and applied in 20-μL doses. This procedure provided 0.1, 0.5 or 1.0 NE for each treatment. Equal proportions of male and female fifth-instar exuviae were used in these preparations. Two batches of the extractions for each of the three doses were made for this experiment, which were used on two different weeks, such that half the replications were from either series of extractions. For this experiment, the insects were again used 2 days after feeding but only for four successive days per cohort.

Twenty behavioural assays were performed each day with ten groups of males and ten groups of females. On each of the first 3 days of the experiment, each group was exposed to a choice involving the control versus a single dose of extract. The order in which the doses were presented to the groups was randomized. One week later, new groups of *C. lectularius* were organized, and the procedure was repeated for another 3 days, throughout which these groups were also exposed to each of the three possible treatment doses. For both male and female groups, this procedure provided 20 replications of the choice test at each dosage. Half of both the male and female groups were virgins and the other half nonvirgins.

Experiment 3: Two-way choice by male and female adults in response to fifth-instar exuvial extracts of either sex

Insect groups were presented with filter paper refuges having extracts from either male or female fifth-instar exuviae. The extracts were prepared such that there were 50 exuviae mL^{-1} dichloromethane. Applications of 20-μL doses of these solutions provided 1.0 *C. lectularius* equivalents on each refuge. Two different batches of male extracts ($♂^A$ and $♂^B$) and female extracts ($♀^A$ and $♀^B$) were made and kept separately. This allowed the assessment of whether any potential preferences for male or female extracts was consistent across different batches of extracts.

Sixty behavioural assays were performed over 3 days. Within each day, ten groups of adult males and ten groups of adult females were tested. These groups were tested for response to refuges treated with either a male or female extract. The treatments were randomized such that each group of bugs was exposed to different combinations of extracts ($♂^A$ or $♂^B$ versus $♀^A$ or $♀^B$). Again, half of both the adult male and female groups assayed were virgins and the other half were nonvirgins. The bugs were fed 2 days before the experiment.

Experiment 4: Adult male responses to fourth-instar exuviae extracts

Adult male groups were presented with filter paper refuges having a solvent control versus an extract of fourth-instar exuviae. The extract was prepared at a concentration of 50 exuviae mL^{-1} dichloromethane and applied in 20-μL doses to provide 1.0 NE. According to Usinger (1966), fifth-instar exuviae are approximately 1.5 times longer than fourth-instar exuviae, and approximately 1.3 times wider (averaged from head, pronotum, mesonotum, metanotum and abdomen measurements). Assuming the surface area is proportional to the length multiplied by the square of the width, the surface area of a fifth-instar exuvia would be approximately 2.5 times that of a fourth instar. This means that 1 NE of the fourth-instar extract is likely to have a similar amount of surface compounds as 0.4 NE of a fifth-instar extract. Again, the *C. lectularius* were fed 2 days before the experiment.

Thirty behavioural assays were performed over 2 days. Each day, 15 groups of six adult males were tested for responses to refuges treated with dichloromethane or extract of fourth-instar exuviae. Ten of the groups of adult males included only virgin males and the other five groups consisted of nonvirgins.

Statistical analysis of arrestment patterns

Preference was tested with respect to the number of *C. lectularius* present in either refuge or in areas outside the refuges. Only data from the measurements at 20 h were used to assess statistical significance. This time point was considered to be the most biologically relevant because it provided location choices after a long undisturbed period, during which the insects were able to fully acclimatize to the arena without interference. The most common multinomial models (e.g. loglinear analysis) to determine location preference could not be applied because the test insects often did not act independently (i.e. they were gregarious), making the chi-square or *G*-test statistics from such a model invalid. Instead, a Bayesian generalized linear mixed model extension to multinomial loglinear models was applied using R software (R Development Core Team, 2009) with the MCMCglmm add-on package (Hadfield, 2010). This model accommodates random effects (cohort) and allows for the overdispersion (as a result of gregariousness). Because the output of the MCMCglmm package produces a posterior distribution for each of the parameters (rather than a conventional *P*-value), a preference was declared only when a calculated *t* statistic [$\text{mean}(\delta_i)/\text{SD}(\delta_i)$, where δ_i is the difference in the (paired) parameter estimates that correspond to the two choices of interest from the *i*th sample of the posterior distributions] was greater than two. This multinomial model, with three locations, allows up to two *a priori* comparisons. The difference between the numbers of insects on the treatment refuge versus the control refuge is the only comparison of interest, and is presented in the results.

The output was also used to create 95% credible (Bayesian confidence) intervals for the mean proportion over all trials at each location. These can be rather wide as a result of cohort-to-cohort differences and should not be used to judge whether location preferences exist. The testing of the latter (given above), similar to paired *t*-tests, uses within-trial differences. Both types of statistics include an effect of overdispersion (i.e. an overdispersion parameter was estimated), resulting from non-independent movement.

Assessing interactions between insects

An index of nonrandom location (NRL) was calculated for each experimental group. This index provides greater

information about gregariousness than the overdispersion parameter. The index is based on a null model assuming that each insect will be found in any of the three areas (either of the two pieces of folded filter paper or elsewhere in the arena) with a probability equal to the overall proportion of insects observed at that location over all the experimental replications. Because of differences in the physical landscape of these areas, it was deemed most relevant to consider a model where a discrete choice was made between the physically and chemically distinct refuge and nonrefuge areas. The *C. lectularius* were found generally either in the folds of the refuges or at the margins of the large filter paper, making it difficult to weight the innate preferences for such areas based on the particular physical dimensions of the spaces.

The model used to develop the NRL index provides expected frequencies for each of seven patterns. For example, all six *C. lectularius* may be in one of the three locations. In another case, three may be in one location and three in another. It is also possible to have two in each of the three locations. All seven possible patterns (ignoring ordering of the numbers within a pattern) are represented as: 0, 0, 6; 0, 3, 3; 2, 2, 2; 0, 1, 5; 0, 2, 4; 1, 1, 4; and 1, 2, 3.

As an example of how the index works, it is assumed that the average number of *C. lectularius* in each of the zones is two across the experiment. However, in an individual trial, the *C. lectularius* tend to be found in groups of five or six in one zone (with no preference shown for zone because the average is two across the experiment). This would result in a greater number of *C. lectularius* than expected in the 0, 0, 6 and 0, 1, 5 patterns compared with how many *C. lectularius* would be in these two patterns if they were randomly distributed. Without clustering, the 2, 2, 2 and 1, 2, 3 configurations are expected to be most common, although there would still be some trials displaying the other patterns (with just a few expected to show the 0, 0, 6 pattern).

After calculating the expected frequencies for each of the seven patterns, based on animals distributing themselves independently (although possibly with location preferences) among the three locations, a natural index of NRL for these experiments is the familiar chi-square statistic [sum of $(\text{observed}-\text{expected})^2/\text{expected}$, where this expression is summed over the seven patterns]. Either over- or under-dispersion can contribute to this statistic. Although the NRL index was used to gauge primarily how far from independence the insects behaved, this chi-square statistic can also form the basis for a formal hypothesis test. Given that the statistic is based on seven distribution patterns, there are six degrees of freedom. When a Bonferroni correction is used to account for the 48 indices that were calculated, a (very conservative) threshold of 22.36 indicated significant nonrandom movement at $\alpha = 0.05$ (experiment-wise error rate).

Results

Over the four experiments, there was no evidence of differences in the results obtained with respect to which preparation of the extractions was used. There were also no differences between the behavioural responses of virgin and nonvirgin adults. Because there were no differences in these factors, they were not included in any statistical models.

Among the groups tested for preference for 0.2 NE fifth-instar extracts, only the adult groups showed a tendency to reside on the treated paper (Fig. 2). When first- and second-instar nymphs were used, many *C. lectularius* stayed out of the refuges throughout the experiment, despite some movement toward the refuges (Fig. 2a). Greater proportions of *C. lectularius* entered the refuges when older-instar nymphs were used but, again, no preference for either refuge was expressed (Fig. 2b, c). The adult males showed a significant tendency to inhabit the extract-treated refuge in each choice test, whereas very few of the bugs remained outside a refuge (Fig. 2d). This difference between the number of males on the treated versus control refuges was significant at the 20-h interval. Females did not show a statistically significant preference, although, on average, there were slightly more females in the extract-treated versus control refuges (Fig. 2e). In groups of mixed nymphs and adults, there was no preference for either the extract-treated or control refuges, although the *C. lectularius* tended to be found in the refuges 2 h into the experiment (Fig. 2f). The estimates of cohort-to-cohort variation were two- to three-fold greater in this experiment than in subsequent experiments, perhaps as a result of changes in the methodology from this point forward including the use of more cohorts over shorter time durations after feeding.

In most cases, the various groupings had significantly high NRL indices, indicating gregariousness, which increased over time (Fig. 3). This was particularly true for all groups that included nymphs (Fig. 3a–c, f), where there were always large increases in the NRL indices during the period when the insects were undisturbed overnight. In all cases where this index was significant, a much greater number of *C. lectularius* than expected was distributed as five or six insects in one location (details not shown). Although the first- and second-instar nymphs do not have high NRL indices for the first 2 h, the NRL indices were significant at 20 and 21 h (Fig. 3a). Indices were particularly high for the groups of nymphs composed of later instars (Fig. 3b, c). The data from the present experiments depicted over-dispersion overwhelmingly, implying that the insects were found in larger groups than expected by chance after the general positional preference was taken into account. In other words, all occurrences of very high NRL index arose when five or six insects tended to be found together in one area more often than expected.

Males showed a pattern much different from these groups, with the NRL indices decreasing and becoming statistically insignificant over time (Fig. 3d). Females were also different from the groups that included nymphs because the NRL index decreased overnight, although the values for the index remained relatively high and statistically significant throughout the experiment (Fig. 3e). Under-dispersion occurs if insects are distributed more uniformly than expected under random movement. Some data sets involving adult males showed such a tendency because there were four males in the treated refuge and two in the other more often than predicted by chance, and fewer in the 0, 0, 6 and 0, 1, 5 patterns. This suggested the

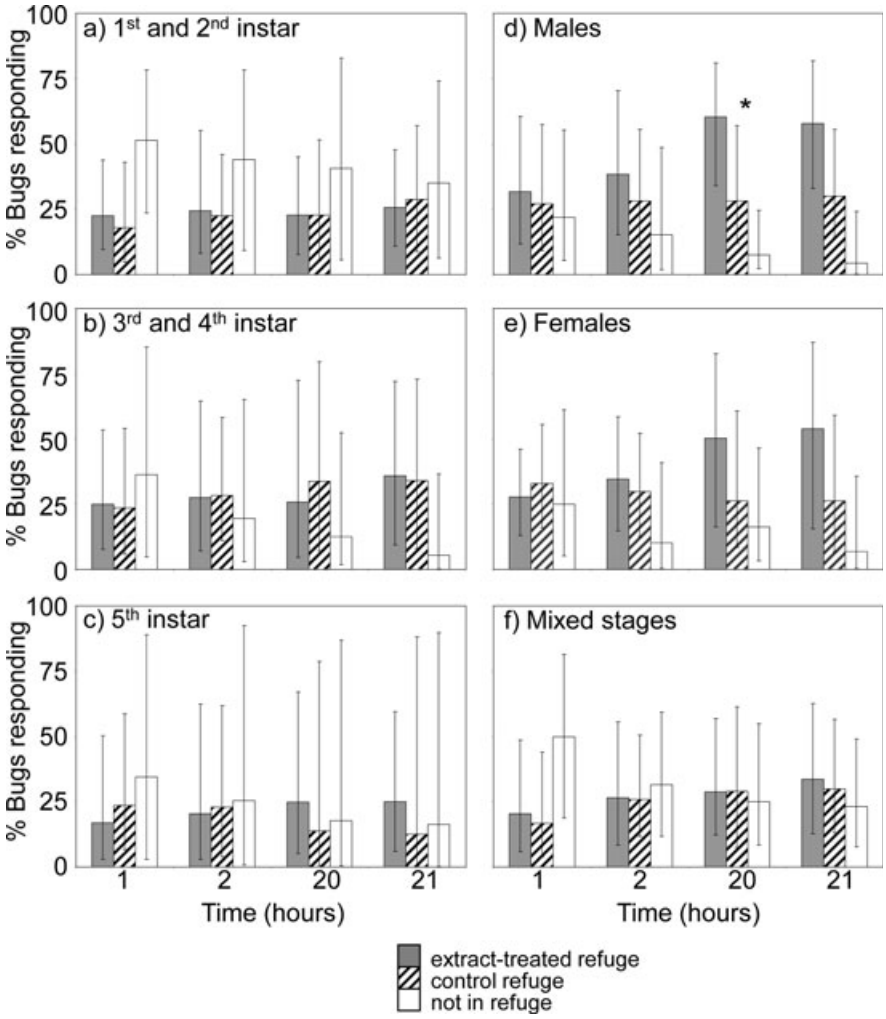

Fig. 2. Percentages of *Cimex lectularius* of various instars or adult sexes found in the fifth-instar extract treatment refuge, a dichloromethane control refuge, or not in a refuge, at four measurement times (median and 95% confidence interval). The extract treatments consisted of 0.2 nymphal equivalents from unsexed fifth-instars. Six *C. lectularius* of a particular type were tested per arena as indicated. The mixed stage group included one male adult, one female adult and one nymph of each instar from two to five ($n = 28$ for each group). *Significant difference between treatment and control refuge at 20 h ($\alpha = 0.05$).

possible avoidance of other males despite a preference for the extract. However, this phenomenon is mathematically difficult to assess because some of the seven distributive patterns cannot be clearly distinguished as representing over- or under-dispersion. In any case, because the NRL in possible cases of under-dispersion was orders of magnitude smaller than in the clearly over-dispersed trials, no attempt was made to resolve this issue further.

For each of the doses of fifth-instar extract used, male groups showed a clear preference for the extract treatment (Fig. 4a–c). For males, the number of *C. lectularius* on the treated paper versus the control paper at 20 h was significantly different, with no indication of an effect of dose. However, at the highest dose, male *C. lectularius* showed some movement from the extract-treated refuge to the control refuge at the very last measurement point. Females showed no clear preference for the extract-treated refuges compared with controls throughout this experiment (Fig. 4d–f). The difference between the numbers of females at the treatment refuge versus the control refuge was not statistically significant at 20 h.

The NRL index patterns for the dose-response experiments again showed lower values for males (Fig. 5a–c) and higher values for females (Fig. 5d–f). The NRL values for males did not rise generally above the significance threshold, with the exception of 2 h at the 0.5 NE dose (Fig. 5b). Although the female indices were high throughout the experiment, they increased in a manner proportional to the dose. At the 0.1 NE dose, the maximum NRL index value was 75 (Fig. 5d). When the 0.5 NE dose was used, the NRL index value reached 136 (Fig. 5e). At 1 NE, the index immediately became very high and reached a maximum of 252 at 20 h (Fig. 5f). In all of the cases involving females throughout this experiment, the

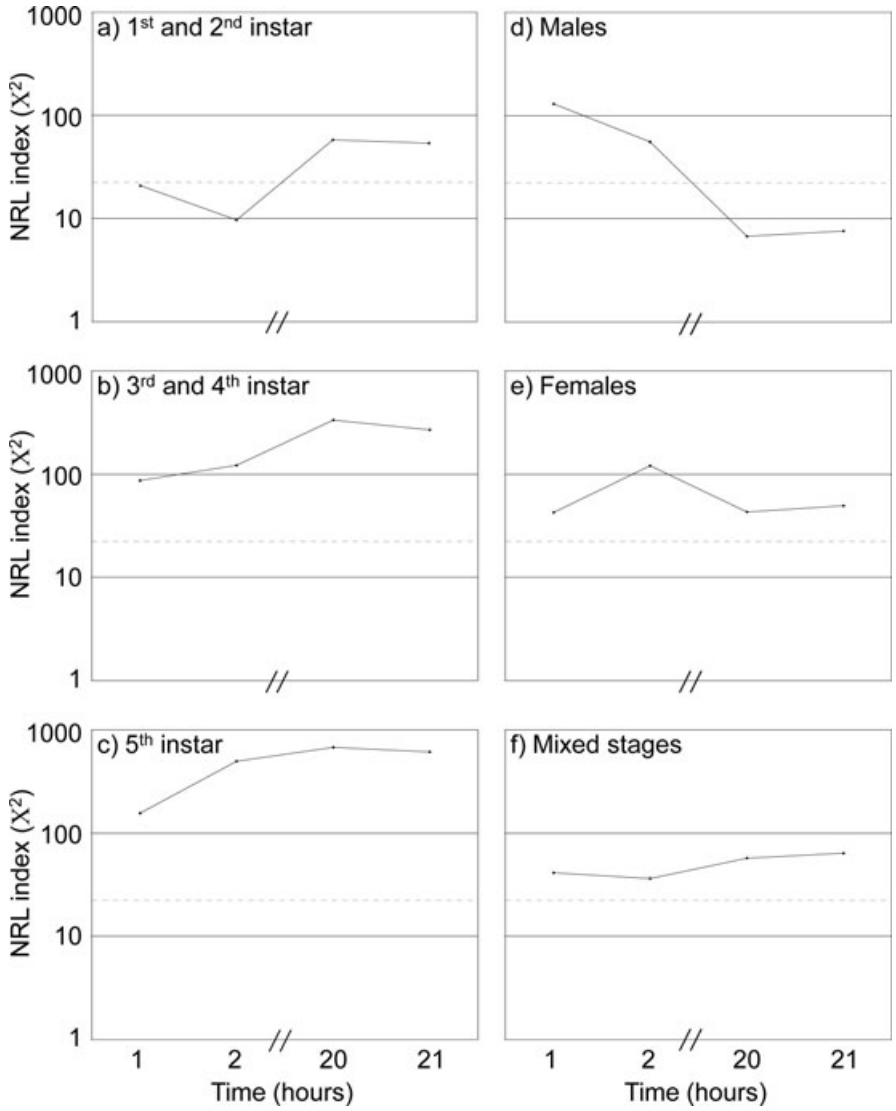

Fig. 3. Nonrandom location index (NRL) for each group of *Cimex lectularius* after being presented with 0.2 NE from unsexed fifth-instars at four measurement times. Six *C. lectularius* of a particular stage or sex were tested per arena as indicated. The mixed stage group included one male adult, one female adult and one nymph of each instar from two to five ($n = 28$ for each group). The threshold for significance of the index at $\alpha = 0.05$ is included as a straight broken line (all indices are χ^2 with 6 d.f.; Bonferroni correction employed).

frequent observation of groups of five or six females in a single location (i.e. indicating over-dispersion) contributed heavily to this index (details not shown).

Neither males nor females showed any preference for male versus female fifth-instar extracts (Table 1). When presented with this choice, most *C. lectularius* went into one of the two refuges, as in the previous experiments, but similar percentages went into either of the two refuges. Likewise, the male adults did not show a preference for fourth-instar extracts over controls as they did for fifth-instar extracts (Table 1). Both of these final experiments showed sex-specific temporal patterns for the NRL indices similar to those shown in the previous experiments, and thus they are not documented here.

Discussion

Male *C. lectularius* prefer filter paper treated with dichloromethane-soluble elements of fifth-instar exuviae, at a threshold somewhere below 0.1 NE. This arrestment tendency does not increase at higher doses. Furthermore, the males distribute themselves, from one experimental trial to the next, in a pattern that is consistent with independent movement. Thus, although males are arrested in refuges treated with the extracts of exuviae from fifth-instar nymphs, their location preference does not appear unduly influenced by other males.

Unlike the adult male groups, no evidence is found that adult females or nymphs have a significant preference for

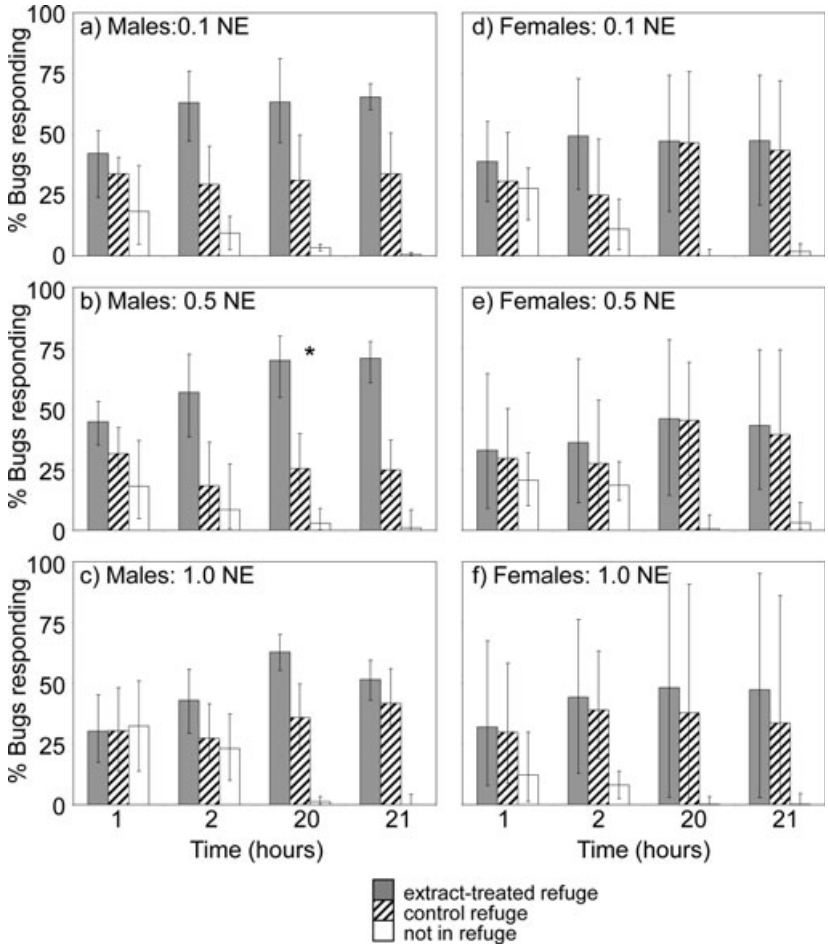

Fig. 4. Percentages of *Cimex lectularius* from male and female groups found in a fifth-instar extract treatment refuge, control refuge or not found in a refuge when treatment doses were varied at four measurements (median and 95% confidence interval). The dichloromethane extracts for treatments were all derived from equal proportions of male and female fifth-instar exuviae ($n = 20$ for both sexes at each dose). *Significant difference between treatment and control refuge is significant at 20 h [$\alpha = 0.05$ for the particular dose indicated (and if data for males at all doses are combined)]. NE, nymphal equivalent.

extract-treated refuges. However, certain aspects of the behaviour of these groups may have limited the power of the assay to detect such preferences. For example, the youngest nymphs are less likely to be found in the refuges than other groups, such as adult males or females, providing a lower power for the comparisons between the refuges. There are many possible reasons why the young nymphs are not often found in the refuges, including age-specific differences in response to the physical dimensions of the assay. Arrestment may have occurred for the younger *C. lectularius* nymphs had the arena been constructed differently. A recent study documents the arrestment of *C. lectularius* nymphs when exposed to chemicals deposited by conspecifics (Olson *et al*., 2009). Furthermore, the strong tendency for nonrandom movement among the nymphs and females may overwhelm any potential preference for the treated refuge. Thus, it is not clear from the present results that adult males are the only stage with the potential for an arrestment response in response to cuticular extracts.

Given this caveat, it is reasonable to consider that strong sexual conflict (Stutt & Siva-Jothy, 2001) might place selective pressure, on males in particular, to recognize and show preferences for locations where fifth-instar nymphs are emerging into adults. Such locations may help males locate newly-emerging virgin females, even if the assessment of the cuticular compounds is not sex-specific. The lack of response by the males to the fourth-instar extracts provides some evidence that this might be a signal of the presence of mature bugs. Furthermore, the possibility is not precluded that the behaviourally relevant compound is deposited by the adults as they emerge from the nymphal exuviae. Cuticular hydrocarbons, which are abundant in the extracts tested, are well known to function as sex-pheromones in other insects (Liimatainen & Jallon, 2007; Eliyahu *et al*., 2008; Lacey *et al*., 2008; Lelito *et al*., 2009;

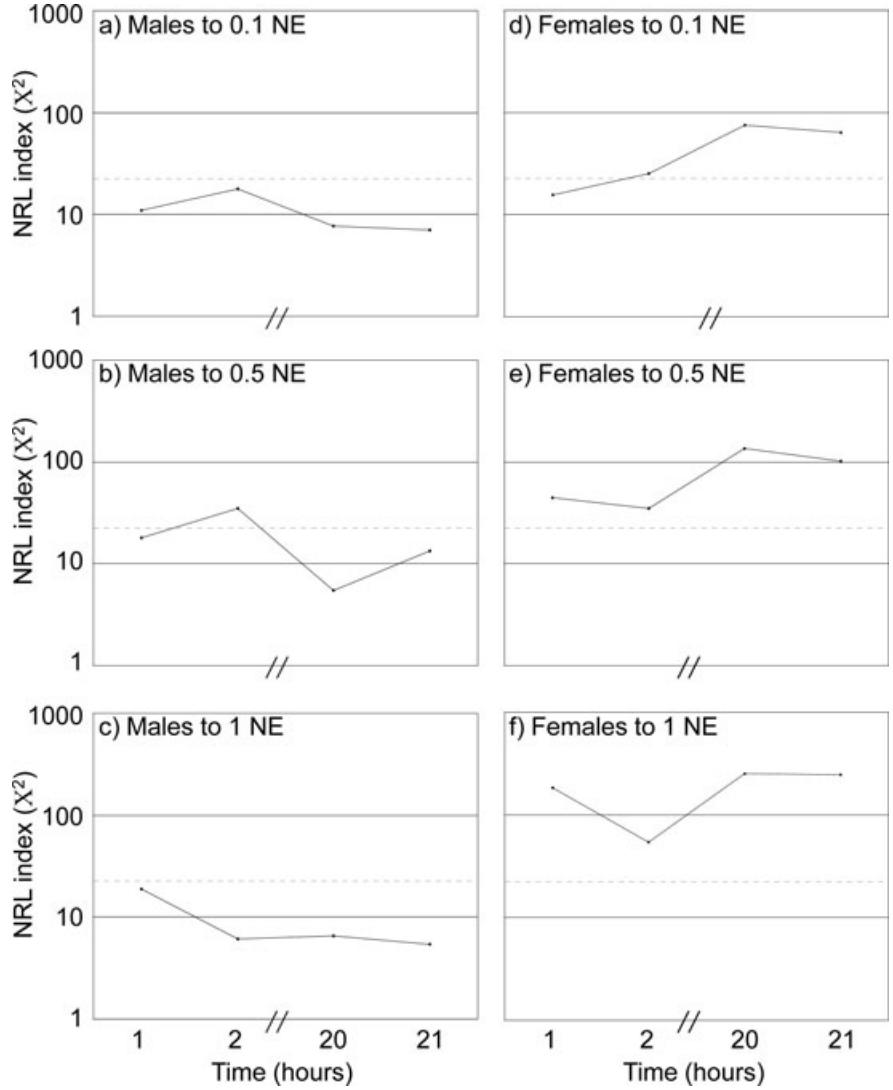

Fig. 5. Nonrandom location index (NRL) for groups of male and female *Cimex lectularius* presented with increasing doses of fifth-instar extract treatment at four measurement times. The dichloromethane extracts for treatments were all derived from equal proportions of male and female fifth-instar exuviae ($n = 20$ for both sexes at each dose). The threshold for significance of the index at $\alpha = 0.05$ is included as a straight broken line (all indices are χ^2 with 6 d.f.; Bonferroni correction employed). NE, nymphal equivalent.

Table 1. Proportion (median and 95% confidence interval) of *Cimex lectularius* found at either of the two chemically treated refuges or elsewhere in a choice arena after 20 h of exposure.

	Refuge 1	Refuge 2	
	Male fifth-instar extract	Female fifth-instar extract	Nonrefuge areas
Males	0.47 (0.38, 0.57)	0.46 (0.36, 0.56)	0.07 (0.04, 0.12)
Females	0.49 (0.36, 0.63)	0.49 (0.36, 0.62)	0.02 (0.01, 0.05)
	Refuge 1	Refuge 2	
	Fourth-instar extract	Solvent control	Nonrefuge areas
Males	0.48 (0.38, 0.59)	0.46 (0.36, 0.56)	0.06 (0.03, 0.12)

Oppelt & Heinze, 2009). Thus, it would not be surprising for such compounds to provide a similar function in *C. lectularius*.

By comparison with males, females react to the extract of nymphal exuviae by increased clustering in nonspecific areas of the bioassay chamber, made clear by the dose-dependent effect of the extract on gregarious behaviour. Such an effect clearly does not occur in males, as judged by the effect of extract dose on the NRL index of males. It is possible that the gregarious behaviour observed in nymphs in the first experiment is similarly induced by the extract. Because the chemical nature of the observed signals has not yet been investigated, it is not known whether the same semiochemicals might induce both female gregariousness and male arrestment. Such signal parsimony is not uncommon

in insect pheromone systems (Blum, 1996). For example, in honey bees, the same compounds stimulate different physiological pathways, evoking both releaser and primer responses (Grozinger *et al*., 2007).

The biological utility of such a dose-dependent and nonlocation specific induction of gregariousness is of interest. The presence of shed exuviae of other *C. lectularius* might indicate that they have located an area that conspecifics have used as a successful refuge in the past. The greater the frequency of such contacts, the more likely it would be that a large aggregation either exists or has existed in this location, indicating the suitability for *C. lectularius* to become gregarious and aggregate. Conversely, a strong arrestment response upon encountering another conspecific or a single exuvia might not always lead the *C. lectularius* to a favourable location and may be less efficient in promoting clustering. Although the gregariousness that is induced in female *C. lectularius* does not result in the pronounced physiological changes caused by primer pheromones in truly social insects (Le Conte & Hefetz, 2008), from a functional perspective, the signal is similar in that it changes how the insects interact with each other. It may be fruitful therefore to consider this system further in an attempt to understand the biochemical and physiological mechanisms of releaser and primer pheromone responses in this species.

Because the arrestant described comprises a dichloro methane-soluble element from the cuticle, it is likely to be chemically different from the methanol-soluble substrate-deposited material described in other studies involving *C. lectularius* (Levinson & Bar Ilan, 1971; Parashar *et al*., 2003; Siljander *et al*., 2007). Considering also the attraction to airborne compounds that emanate from aggregations of *C. lectularius* (Siljander *et al*., 2008), the mechanism by which attraction, arrestment and aggregation occurs in wild populations of bed bugs may be even more complicated. Thus, significant additional studies are required to understand fully both the nature and context of the cuticular signals that are described in the present study. These semiochemicals, as well as other potentially similar signals, may be useful for the design of bed bug trapping devices.

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